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Fluorescent "In Situ" Hybridization of Hepatitis C Virus RNA in Peripheral Blood Mononuclear Cells From Patients With Chronic Hepatitis C

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Although the liver is the main target for hepatitis C virus (HCV) infection, HCV RNA of positive and negative polarity has also been detected in peripheral blood mononuclear cells (PBMCs) by polymerase chain reaction. However, no data have been published on the relationship between the number of HCV-infected PBMCs and serum viremia levels. To address this issue, PBMC samples from 20 patients with chronic hepatitis C were examined by fluorescent "in situ" hybridization. Serum viremia levels and viral load in infected PBMC were measured using the Amplicor Monitor test. HCV was detected in all PBMC samples corresponding to the HCV-positive patients. Fluorescent signals were found mainly in the cytoplasm of the cell. The percentage of positive cells ranged from 0.08% to 4%, with a statistical correlation with the viral load in PBMC ($r = 0.69$; $p = .001$) but not with the serum viremia levels ($r = 0.23$). It was demonstrated that HCV infection of PBMCs is a common feature of HCV chronic carriers. The results suggest that HCV infection of PBMCs does not contribute significantly to HCV viremia. ***J. Med. Virol. 60:269–274, 2000.***

KEY WORDS: fluorescent signals; viral load; viremia levels; polymerase chain reaction

INTRODUCTION

Hepatitis C virus (HCV) is the main agent of post-transfusion acute and chronic non-A non-B hepatitis [Kou et al., 1989]. The HCV genome consists of single-stranded RNA of positive polarity of about 9.4 Kb in length [Kato et al., 1992].

Although the liver is the main target for HCV infection and replication [Fong et al., 1991; Takehara et al., 1992], HCV RNA of positive and negative (the putative replicative intermediate) polarity has been also detected in peripheral blood mononuclear cells (PBMCs) by the polymerase chain reaction [Wang et al., 1992; Zignego et al., 1992; Bartolomé et al., 1993]. It has been argued that the detection of negative polarity HCV RNA may be a laboratory artifact, and that HCV does not infect PBMCs [Lanford et al., 1995; Lerat et al., 1996; Laskus et al., 1997]. However, the fact that hepatitis could be induced successfully in chimpanzees by inoculating plasma leukocytes from a patient with non-A, non-B hepatitis would suggest that the virus replicated in vivo in extrahepatic tissues [Hellings et al., 1985].

Furthermore, using "in situ" hybridization and immunofluorescence, the presence of the negative HCV RNA strand and viral-encoded antigens in PBMCs, respectively, has been reported [Moldvay et al., 1994; Sansonno et al., 1996]. These findings suggest that HCV not only infects PBMCs but can form viral particles and release them into the circulation. However, no data have been published on the relationship between the number of HCV-infected PBMCs and serum viremia levels. A specific and sensitive fluorescent "in situ" hybridization method was developed to correlate the percentage of HCV-infected PBMCs with the viral load in these cells and in serum.

PATIENTS AND METHODS

Patients

PBMC samples isolated from 20 anti-HCV-positive and serum HCV-RNA-positive patients were examined in this study. All patients had a histologically proven chronic hepatitis according to international criteria [Desmet et al., 1994] and had elevated alanine aminotransferase (ALT) levels. None had received antiviral or immunosuppressive therapy before the study.

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As negative controls, PBMCs from 10 healthy blood donors with normal ALT levels, who were anti-HCV negative and without HCV RNA detectable in serum were used.

Methods

PBMCs were separated from 20 ml of heparinized whole blood by centrifugation in a Ficoll-Hypaque gradient (Ficoll-Hypaque; Pharmacia, Uppsala, Sweden). The phase corresponding to the mononuclear cells was recovered and washed three times in phosphate-buffered saline (PBS) solution. The cells were then resuspended in PBS at a final concentration of 10^7 cells/ml in PBS.

For fluorescent "in situ" hybridization (FISH), 10^6 mononuclear cells were centrifuged at 1,200 rpm for 10 min, resuspended in freshly prepared 4% paraformaldehyde in PBS and then fixed during 10 min at 4°C. After fixation, the cells were pipetted onto Vectabond (Vector, Burlingame, CA)-treated slides (10^5 cells per slide). The slides were air dried and then washed three times in PBS and dehydrated through a graded series of ethanol dilutions (30–70%). The slides were stored in 70% ethanol at 4°C.

Production of the Probe

A polymerase chain reaction (PCR) product corresponding to the complete 5′non-coding region of the HCV genome was cloned directly in the pCR 1000 vector (Invitrogen, San Diego, CA). The HCV-cDNA was excised from the plasmid by HindIII/XbaI digestion and the fragment was gel-purified using the Geneclean kit (Bio 101, Vista, CA). The purified DNA was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) by nick translation (Nick Translation kit, BRL Life Technologies, Gaithersburg, MD). After ethanol precipitation, the labeled probe was redissolved in the hybridization mixture (50% deionized formamide, 10% dextran sulfate, 100 μg/ml sonicated salmon sperm DNA, and 250 μg/ml tRNA in 2×SSC) at a final concentration of 100 ng in 20 μl and stored at −20°C.

FISH

Before FISH, the slides were dehydrated by successive incubation in 70%, 90%, and 100% ethanol and then rehydrated by a series of ethanol dilutions. The slides were then rinsed in PBS and postfixed in freshly prepared 4% paraformaldehyde in PBS for 20 min at room temperature. The cells were digested subsequently with 1 μg/ml of proteinase K (GIBCO, BRL) in 20 mM Tris HCl pH 7.4, 2 mM $CaCl_2$ at 37°C for 7 min. After digestion, the slides were rinsed in PBS for 5 min, refixed in 4% paraformaldehyde for 5 min, dipped in distilled water, dehydrated through a series of ethanol dilutions (30–100%) at −20°C, and allowed to dry for at least 2 hr.

The probe (20 ng per slide) was denatured for 5 min at 90°C, quenched on ice, and then applied to the slides under coverslips sealed with rubber solution. The hybridization was carried out at 50°C for 16 hr in a humid chamber.

After hybridization, the slides were washed at 42°C in 2×SSC, 0.5×SSC, and 0.1×SSC (15 min each). The digoxigenin-labeled hybrids were detected with fluorescein isothiocyanate conjugate (FICT) (Boehringer Mannheim). The intensity of the signals were amplified with three antibodies (antidigoxigenin-mouse; antimouse-Ig-digoxigenin; antidigoxigenin-FICT, 50 ng each) using the Fluorescent Antibody Enhancer Set for DIG Detection kit (Boehringer Mannheim). The slides were counterstained with propidium iodide/antifade (0.6 μg/mL) (ONCOR, Appligene; Heidelberg, Germany).

The specificity of the hybridization signals was assessed by pretreatment of the slides with RNAse A (20μg/ml) before hybridization, hybridization with the PCR 1000 vector alone labeled with digoxigenin, and omission of the probe in the hybridization mixture.

To confirm that the hybridization signals corresponded to intracellular HCV RNA and not to particles attached to the cell membrane, different experiments were performed: (a) omission of the proteinase K digestion of the samples to avoid penetration of the labeled probe inside the cell; (b) treatment of the samples with 0.05% trypsin, 0.02% EDTA for 20 min at 37°C, followed by 5 mg/ml RNase A (Boehringer Mannheim) treatment for further 15 min at 37°C as described previously [Cribier et al., 1995].

Finally, PBMCs from a healthy donor without HCV infection markers were incubated with 50 μl of HCV-positive serum (2×10^6 genome copies/ml) for 10 min at 37°C. After five washes with PBS, the cells were processed for the FISH. At least 10^3 cells per sample were counted to obtain the percentage of positive cells.

As a control of the sensitivity of the technique, six liver biopsies from patients with histologically proven chronic hepatitis C and with viremia levels ranging from 1.2×10^4 to 3.6×10^6 HCV genome copies/ml and two liver samples from two patients with chronic hepatitis B without HCV infection markers were also analyzed by FISH as described above for the PBMCs. The images were visualized and captured with a NIKON Eclipse E-400 microscope.

HCV RNA Quantitation in Serum and PBMCs

Serum HCV RNA quantitation was carried out using the Amplicor HCV Monitor Assay (Roche Diagnostic System, Branchburg, NJ). HCV RNA was quantitated in duplicate in PBMC samples (10^6 cells) suspended in 100 μl of PBS using the Amplicor HCV Monitor test with the modifications described previously by Martín et al. [1998]. Using this modification the results are expressed as HCV RNA copies/1.5 μg of total RNA.

Statistical Analysis

The Pearson test and the Spearman's rank correlation coefficient were used for correlations. All statistical analyses were carried out using the SPSS package (SPSS for windows release 6.0 SPSS, Chicago, IL).

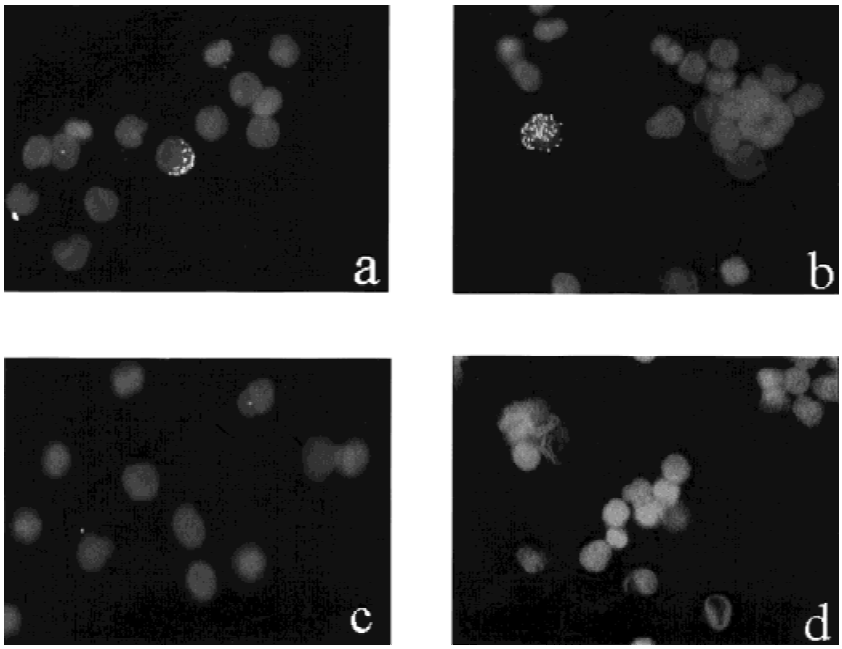

Fig. 1. Fluorescent "in situ" hybridization (FISH) in peripheral blood mononuclear cells (PBMC) from hepatitis C virus (HCV)-infected patients. The fluorescence signals are located in cytoplasm **(a)** and in both nucleus and cytoplasm **(b)** of the PBMC from HCV-infected patients. No signals were detected when the hybridization was carried out with an unrelated probe **(c)** or in PBMC from anti-HCV-negative, serum HCV-RNA-negative controls hybridized with HCV-derived probe **(d)**. Counterstained with propidium iodide. Magnification = ×650.

RESULTS

FISH Detection of HCV RNA in PBMC

Fluorescent hybridization signals were detected in all the PBMC samples corresponding to the HCV-RNA-positive patients. No hybridization was observed in PBMCs from the 10 healthy controls without HCV markers (Fig. 1). The percentage of positive cells ranged from 0.08% to 4% (Table I).

"In situ" hybridization signal specificity was demonstrated by the lack of positive signals when the slides were hybridized with the vector alone and when the probe was omitted in the hybridization mixture (Fig. 1). Furthermore, the positive signals were abolished with the RNAse A treatment before FISH.

Fluorescent signals were found mainly in the cell cytoplasm, although nuclear signals were also observed in scattered cells. The intracellular localization of the hybridization signals was confirmed by the lack of signals when the proteinase K digestion step was omitted and by the fact that the trypsin/EDTA and RNase A digestion of the samples before hybridization did not change the pattern of the positive signals (Fig. 2). Furthermore, no hybridization signals were detected in the PBMCs from a healthy donor that were incubated previously with a positive serum.

No statistical correlation was found between the percentage of HCV-infected cells and the known time of HCV infection in each patient.

The percentage of FISH-positive hepatocytes in liver biopsies ranged from 15% to 70%. No positive signals were detected in the liver biopsies from the two patients with chronic hepatitis B (Fig. 3).

HCV Quantification in PBMC

HCV RNA could be quantified in all PBMC samples tested. Overall, the viral load in all samples tested ranged from 18 to 3,060 genome copies/1.5 μg of total RNA (Table I). The statistical analysis showed that there was a positive correlation ($r = 0.69$; $p = .001$) between the percentage of FISH-positive cells and the viral load in these cells.

HCV Quantification in Serum

The viral load in serum samples ranged from 5×10^3 to 8×10^6 genome copies/ml (Table I). No statistical correlation was found between the percentage of FISH-positive cells and the serum viral load ($r = 0.23$). There

TABLE I. Percentage of FISH-Positive PBMC, Cellular Viral Load, and Serum Viremia Levels in Study Patients*

Patient	PBMC viral load genome copies/ 1.5 μg total RNA	Serum viral load genome copies/ml	% infected cells
1	3060	8182741	4%
2	644	5766	2.63%
3	601	2071394	2.57%
4	317	19997	2.1%
5	258	1507246	3.1%
6	176	475695	1.46%
7	153	67079	1.18%
8	147	337582	1.59%
9	140	1392491	1.56%
10	109	140177	1%
11	103	359745	2.2%
12	70	1288951	1.97%
13	56	984721	0.5%
14	50	5554674	1.3%
15	36	2516923	1.9%
16	32	68369	1%
17	29.4	2537143	0.7%
18	23	5584906	0.08%
19	20	1890956	0.5%
20	18	108000	0.1%

*FISH, fluorescent "in situ" hybridization; PBMC, peripheral blood mononuclear cells.

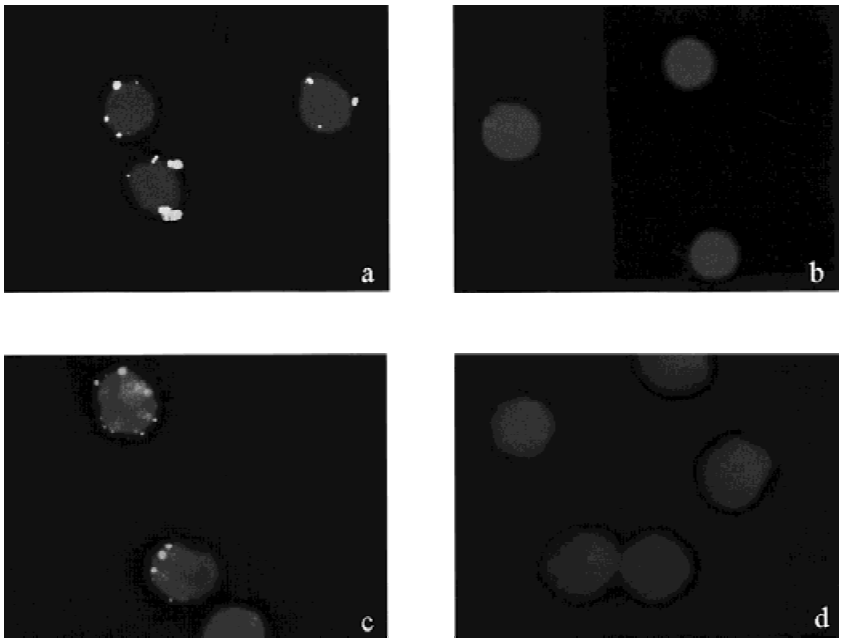

Fig. 2. Demonstration of the intracellular localization of fluorescent "in situ" hybridization (FISH) signals. Peripheral blood mononuclear cells (PBMC) from a hepatitis C virus (HCV) chronic carrier were hybridized using the standard protocol **(a)**; without proteinase K pretreatment before FISH **(b)**; or pretreated with trypsin and RNase A **(c)**. Lack of FISH signals in PBMC form a healthy donor incubated 10 min with HCV-positive serum before FISH **(d)**.

was a positive correlation between the serum and the PBMC viral load ($r = 0.60$; $p = .005$).

To assess whether the percentage of FISH-positive cells or the amount of HCV RNA in PBMCs or in serum were related with the biochemical or histological activity of the disease, serum ALT levels and the histological score were analyzed. Neither ALT levels nor the histological activity index correlated significantly with the above parameters (data not shown). Finally, no statistical correlation was found between the percentage of FISH-positive cells and the known time of HCV infection in each patient.

DISCUSSION

Although HCV RNA of positive and negative polarity has been detected in PBMC by PCR [Wang et al., 1992; Zignego et al., 1992; Bartolomé et al., 1993], "in situ" hybridization [Moldvay et al., 1994; Sansonno et al., 1996], and "in situ" PCR [Muratori et al., 1996], there are no data concerning the contribution of HCV replication in these cells to HCV viremia levels. To study this issue, the percentage of HCV-infected cells from patients with chronic hepatitis C and studied the correlation with the viral load in serum and in PBMCs were investigated. For this purpose, a FISH method that has not been described previously for the detection of HCV in PBMCs was developed. The results were compared with those obtained previously by using "in situ" PCR and isotopic and non-isotopic "in situ" hybridization methods.

In the present study, HCV RNA was detected in the PBMCs of all the 20 patients included in the study, whereas no hybridization signals were observed in the 10 healthy controls without HCV markers. Furthermore, the specificity controls (RNAse treatment, hybridization with the vector alone, etc.) provided the expected results demonstrating the specificity of the technique. In addition, the results obtained when the proteinase K pretreatment was not carried out and when the samples were digested with trypsin and RNase A before hybridization confirm the intracellular localization of the hybridization signals.

The percentage of HCV positive cells found in this study ranges from 0.08% to 4%. This percentage is higher than that reported by Moldvay et al. [1994] (0.01–0.03%) but is similar to that described by Sansonno et al. [1996] (0.1–4%) and by Muratori et al. [1996] (0.2–8.1%). These results show that the sensitivity of the method is similar to the non-isotopic "in situ" hybridization [Sansonno et al., 1996] and to the "in situ" RT-PCR [Muratori et al., 1996]. Furthermore, when the FISH technique was applied to liver biopsies, the percentage of positive hepatocytes ranged from 15% to 70%, which is similar to that described recently [Agnello et al., 1998]. This result also confirms the sensitivity of the technique. On the other hand, the FISH method does not have the problem of false-positive results associated with the PCR and when digital image analysis is applied to the hybridization signals [Gosálvez et al., 1998] it is possible to quantitate the intracellular viral load. Another advantage is that the coexistence of different viruses (i.e., HCV and GBV-C/hepatitis G virus) in the same cell or the influence of HCV infection in the expression of cellular genes can be determined by using different color fluor labels for different probes. This cannot be performed with isotopic or non-isotopic "in situ" hybridization techniques.

A significant correlation was found between the percentage of FISH-positive PBMCs and the cellular viral load but not with the serum viremia levels. This finding suggests that HCV infection of the PBMC does not contribute significantly to serum HCV viremia. In this

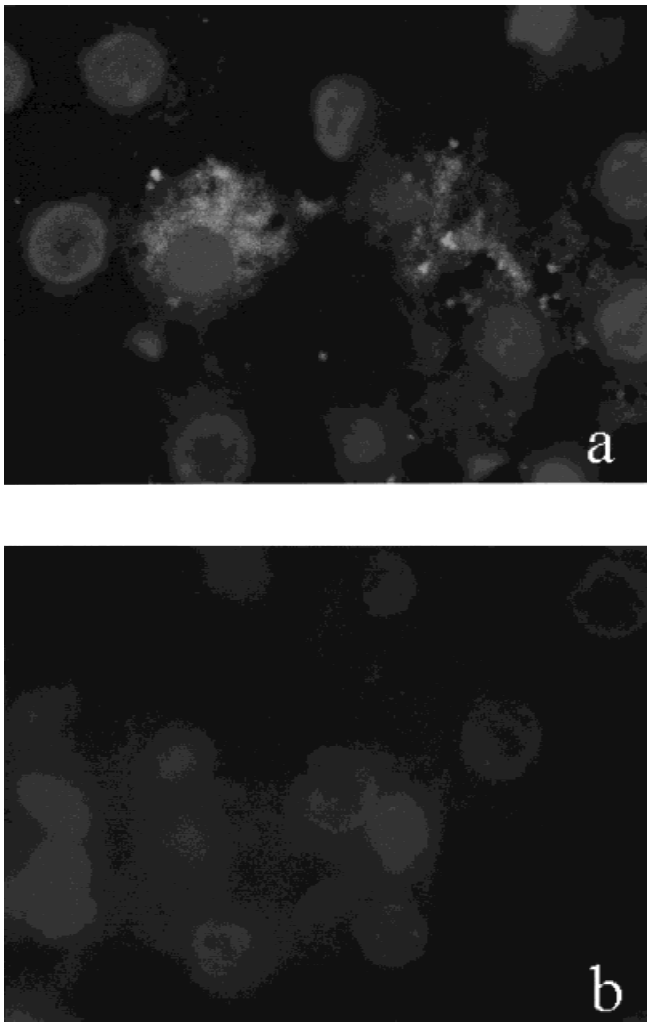

Fig. 3. Fluorescent "in situ" hybridization (FISH) in the liver biopsy from a patient with chronic hepatitis C **(a)** and from a negative control **(b)**.

regard, in severe combined immunodeficiency mice infused with HCV-infected PBMC from human carriers [Bronowicki et al., 1998], it has been demonstrated that although HCV replicates in these cells, it occurs at a very low levels and thus, the number of HCV particles released to the circulation may be too low to influence the serum viremia levels.

The fact that the viremia in serum correlates significantly with the viral load in PBMCs (but not with the percentage of FISH-positive PBMCs) contradicts this hypothesis. However, there are two possible explanations for this apparent discrepancy. First, these results may imply that the contribution of the HCV infection in PBMCs to the HCV viremia in serum does not depend on the percentage of infected cells but on the amount of HCV RNA in these cells. However, this hypothesis is unlikely because a previous report, in which the presence of HCV RNA in liver examined by "in situ" hybridization was correlated with serum HCV RNA concentration, found that serum HCV viremia depends on the number of infected hepatocytes and not on the relative viral load per infected cell [Gosálvez et al., 1998]. On the other hand, the use of FISH makes it possible to distinguish the presence of HCV RNA in necrotized cells, a problem that cannot be avoided when HCV RNA is quantified in PBMCs and that may influence the results.

Finally, no relationship was found between the percentage of FISH-positive PBMCs or the cellular viral load with ALT levels or the histological activity index, indicating that HCV infection of PBMCs does not seem to affect the severity of the liver disease.

In conclusion, using a sensitive and specific FISH technique, it was demonstrated that HCV infection of PBMCs is a common feature of HCV chronic carriers. The results suggest that HCV infection of PBMCs is not related significantly to circulating HCV viremia. However, the contribution of HCV replication in PBMCs to HCV viremia under special circumstances, such as immunosuppression after orthotopic liver transplantation, cannot be ruled out.

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